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Optimization of Particle Bombardment Conditions for Hybrid *Cymbidium*: Part II

Jaime A. Teixeira da Silva* • **Michio Tanaka**

Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan

Corresponding author: * **jaimetex@yahoo.com**

ABSTRACT

Four factors (delayed selection, osmoticum pre-treatment of plant material, choice of plasmid, age and developmental stage of plant material) affecting β-glucuronidase (GUS) transient and stable expression in protocorm-like bodies (PLBs) and embryogenic callus of hybrid *Cymbidium* Twilight Moon 'Day Light' through particle bombardment were optimized, leading to as much as 100% GUS expression in 24-month old plantlets and up to T_3 generation PLBs following bombardment with pWI-GUS and selection on 50-100 mg/l kanamycin sulphate. The ideal conditions for callus and PLB transformation were: delayed selection (1 week after bombardment), osmoticum pre-treatment of plant material (0.3 M raffinose was better than an equimolar amount of mannitol and sorbitol, i.e. 0.4 M each), choice of plasmid (pWI-GUS showed 6-fold more GUS expression than pBI121), and age and developmental stage of plant material (1-week-old PLBs). The *bar* selector gene could also be inserted using pMSP38. These, as well as our previous results, pave the way for repeatable and systematic transformation of *Cymbidium* hybrids using other genes of interest inserted into plasmid cassettes.

Keywords: orchid, osmoticum, plasmid, protocorm-like body, transgenic
Abbreviations: NAA, α-naphthaleneacetic acid; **PLB**, protocorm-like body; **PGR**, plant growth regulator; **TDZ**, thidiazuron (*N*-phenyl-N-1,2,3-thidiazuron-5'-ylurea); **VW**, Vacin and Went

INTRODUCTION

There are extremely few studies that deal with the genetic transformation of *Cymbidium* through particle bombardment (Yang *et al*. 1999; Chen *et al*. 2007; Teixeira da Silva and Tanaka 2009c). In a previous study (Yang *et al*. 1999), we suspect that since the protocol was not repeatable and since several basic parameters were not clearly defined, but which were clarified within our previous study (Teixeira da Silva and Tanaka 2009c), no other studies using particle bombardment emerged after 1999 for *Cymbidium*, although quite a few were developed for *Dendrobium*, *Phalaenopsis* and *Oncidium*. The only other transformation studies that emerged for *Cymbidium* (Chen *et al*. 2007; Chin *et al*. 2007) and *Cymbidium niveo-marginum* (Chen *et al*. 2006) involved *Agrobacterium*-mediated transformation. The best parameters (already standardized) for particle bombardment of *Cymbidium* callus and PLBs are: microcarrier (gold for both explant types), number of shots (2 for both explant types), rupture disk pressure (1100 and 1350 psi), target distance (6 cm for both explant types), explant pre-culture (3, 6 or 9 days for both explant types) period (Teixeira da Silva and Tanaka 2009).

Transgenes in orchids can be detected or selected using several selectable markers: neomycin phosphotransferase II (*npt*II) and hygromycin transferase II (*hpt*II) genes that require the selection agents kanamycin and hygromycin, respectively, continue to be popular despite their potential health and toxicity hazards. The phosphinothricin acetyltransferase gene, *bar*, and the sweet pepper ferredoxin-like protein (*pflp*) gene have shown potential as selectable markers for orchid transformation, although differences in orchid genotype, explant used, and transformation protocol can significantly affect the efficacy of the selectable marker chosen (reviewed in Chai and Yu 2007). The *bar* gene confers resistance to glufosinate and bialaphos (Murakami *et al*. 1986; Anzai *et al*. 1996). The green fluorescent protein (GFP) gene *gfp* has so far only been used for *Phalaenopsis violacea* and *P. amabilis* (Julkifle *et al*. 2010; Semiarti *et al*. 2010).

Kanamycin, the most commonly used selective agent in orchid transformation studies, conferred by *npt*II, was effective in selecting *Cymbidium*, *Dendrobium*, and *Phalaenopsis* transformants (Kuehnle and Sugii 1992; Anzai *et al*. 1996; Yang *et al*. 1999; Yu *et al*. 2001). Various transformation methods were utilized, such as *Agrobacterium*-mediated transformation for *Phalaenopsis* (Belarmino and Mii 2000; Semiarti *et al*. 2010), *Oncidium* (Liau *et al*. 2003) and *Cymbidium* (Chin *et al.* 2007), and biolistic bombardment for *Dendrobium* (Yu *et al*. 1999; Men *et al*. 2003) or *Oncidium* (Li *et al.* 2005). The *pflp* gene confers resistance to the selection agent *Erwinia carotovora*, which causes soft-rot disease in orchids. It was successfully transformed into *Oncidium* and *Phalaenopsis* (You *et al*. 2003; Chan *et al*. 2005). Three other methods have been used to introduce transgenes into orchids, including, for *Dendrobium*, imbibition of seeds in a solution containing foreign DNA, pollen-tube pathway using ovaries, or electro injection into protocorms (Nan and Kuehnle 1995b). The pollen-tube pathway using ovaries was also successful for *Phalaenopsis* (Hsieh and Huang 1995). However, due to their low TE, they have not subsequently been applied to other orchids, including *Cymbidium*.

Like most orchids, one of the most effective ways of clonally propagating *Cymbidium* is through the culture of protocorm-like bodies (PLBs). Embryogenic callus in *Cymbidium* has been induced either from PLB outer epidermal tissue (Begum *et al*. 1994b; Huan and Tanaka 2004a, 2004b; Huan *et al*. 2004), or inner PLB tissue (Begum *et al*. 1994a) in *Cymbidium* hybrids, or from pseudobulb sections, rhizomes and roots of seedlings of *C. ensifolium*, a terrestrial orchid species (Chang and Chang 1998). PLB formation in *Cymbidium* hybrids using PLB thin cell layers, conventional PLB segments and other explant types has been studied

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Research Note

(Teixeira da Silva and Tanaka 2006) to test the effect of medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a), vessel type (Teixeira da Silva and Tanaka 2009a), gelling agent and other media additives (Teixeira da Silva and Tanaka 2009b) on PLB and callus formation. Of significance is the fact that callus which is induced, when placed on PLB-induction medium, forms PLBs, i.e., embryogenic callus are sub-developed PLBs in a nascent stage, i.e. callus, PLBs and somatic embryos are one and the same developmental concept, at least in *Cymbidium* (Teixeira da Silva and Tanaka 2006).

In this study, we continue to determine the ideal conditions for the successful integration of the β-glucuronidase (GUS) gene (*uid*A) and *bar* gene using particle bombardment through the use of PLBs and embryogenic callus from conventional PLB segments of epiphytic hybrid *Cymbidium* Twilight Moon 'Day Light', a popular hybrid. This serves as a first step to inducing disease resistance in *Cymbidium*, which can automatically be achieved by inserting the *bar* gene, which serves not only as a selector gene, but also as a marker gene. Since the GUS assay was used as a confirmatory assay of transgenic orchids such as *Dendrobium*, *Oncidium* and *Phalaenopsis* following antibiotic selection (Men *et al*. 2003; Liau *et al*. 2003; Liao *et al*. 2004), we too have used it here for hybrid *Cymbidium*.

Other than our previous study on *Cymbidium* hybrid (Teixeira da Silva and Tanaka 2009c), to date, only two studies (Yang *et al*. 1999; Chen *et al*. 2007) have reported on the genetic transformation of *Cymbidium* using particle bombardment. In both cases, hybrids were not used. This study therefore constitutes the second report on the genetic transformation of *Cymbidium* hybrids by particle bombardment.

MATERIALS AND METHODS

Chemicals and reagents

All plant growth regulators (PGRs) were purchased from Sigma-Aldrich (St. Louis, USA) and were of tissue culture grade. All other chemicals and reagents were of the highest analytical grade available and were purchased from either Wako (Japan) or Nacalai Tesque (Japan), unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan), which originated spontaneously from shoot-tip culture on Vacin and Went (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium or VW_{PLB}) every two months on modified VW supplemented with 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA) and 0.1 mg L^{-1} kinetin, 2 g L^{-1} tryptone and 20 g L^{-1} sucrose, and solidified with 8 g L^{-1} Bacto agar (Difco Labs., USA). Callus induction and proliferation medium (VW_{CALLUS}) was similar to VW_{PLB}, except that thidiazuron (TDZ) was used instead of kinetin. All media were adjusted to pH 5.3 with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 mL medium in 100 ml Erlenmeyer flasks, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 μmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally bisected PLB (3-4 mm in diameter) segments, 10 per flask, were used as explants for PLB induction and proliferation and for all experiments. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a) for PLB and callus induction, formation and proliferation.

Particle bombardment conditions

A series of four trials were conducted to select optimal particle bombardment conditions. PLBs or embryogenic callus derived from culture on VW_{PLB} or VW_{CALLUS} medium for 90 or 45 days, respectively were used as the material for bombardment using a Bio-Rad Biolistic PDS-1000/He® particle delivery system. All material tested in these trials was purchased from Bio-Rad and all experimental procedures followed the manufacturer's guidelines and recommendations. For all trials, three two plasmids were tested: pBI121 (Clontech), pWI-GUS or pMSP38 (see Anzai *et al*. 1996 and Anzai and Tanaka 2001 for description) (plasmids kindly provided by Prof. Hiroyuki Anzai). Both pBI121 and pWI-GUS contain the *npt*II and *uid*A genes, the former coding for kanamycin resistance while pMSP38 contains the *npt*II and *bar* genes. In all three plasmids, the transgenes are driven by the CaMV 35S promoter.

Explants were subjected to the following trials:

Trial 1: Effect of delayed selection (immediately after, 1 week after or 2 weeks after particle bombardment).

Trial 2: Effect of osmoticum pre-treatment of plant material (with or without an equimolar amount of mannitol and sorbitol, i.e. 0.4 M each (inspired from Russell *et al*. 1992 and Kemper *et al*. 1996) or 0.3 M raffinose (inspired from Russell *et al*. 1992)). For both osmotica, PLBs were plated on VW_{PLB} medium for 24 h before bombardment and for 24 h following bombardment.

Trial 3: Effect of choice of plasmid (pBI121, pWI-GUS, pMSP38).

Trial 4: Effect of age and developmental stage of plant material (1 day-old callus, 1 week-old callus, 3 weeks-old PLBs, or 2.5 months-old PLBs).

GUS assay

GUS was detected in freshly bombarded explants (PLBs or callus) following incubation overnight at 37°C in a histochemical GUS assay (Jefferson *et al*. 1987). Following incubation, GUS expression was recorded and measured as the number of GUS focal points (GFPs) 72 h after particle bombardment. Explant survival was also checked at the same time intervals. GUS was also checked in T_1, T_2 and T_3 generations of PLBs derived from pBI121 and pWI-GUS, and corresponding to 3-, 6- and 9-month sub-cultures of PLBs.

Kanamycin assay

PLBs derived from any treatment with any of the plasmids (all containing the kanamycin-degrading *npt*II gene) were placed on 50 mg L^{-1} kanamycin sulphate (Sigma-Aldrich) for the first two subcultures (3 months each) and then increased to 100 mg L^{-1} for all subsequent cultures indefinitely. Kanamycin resistance was also checked in T_1, T_2 and T_3 generations of PLBs derived from pBI121 and pWI-GUS, and corresponding to 3-, 6- and 9-month sub-cultures of PLBs.

bar assay

Selection on bialaphos-containing medium followed a similar protocol as used for *Phalaenopsis* by Anzai *et al*. (1996), with modifications. Callus (i.e. immature PLBs) or PLBs derived from particle bombardment with pMSP38 were cultured on VW_{PLB} or VW_{CALLUS} medium containing 5 mg L^{-1} bialaphos (Meiji Seika Kaisha, Ltd., Yokohama, Japan). This level was previously optimized during trials by Teixeira da Silva and Tanaka (2009; data not shown) in a range of 0.1 to 10 mg L^{-1}. Bialaphos resistance was also checked in T_1, T_2 and T_3 generations of PLBs derived from pMSP38, and corresponding to 3-, 6- and 9-month sub-cultures of PLBs.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 20 replicates per treatment for callus ad 60 replicates per treatment for PLBs (one replicate was either a 0.5 cm^2 callus piece or a PLB half-segment placed cut surface down on a single sterilized sheet of Whatman

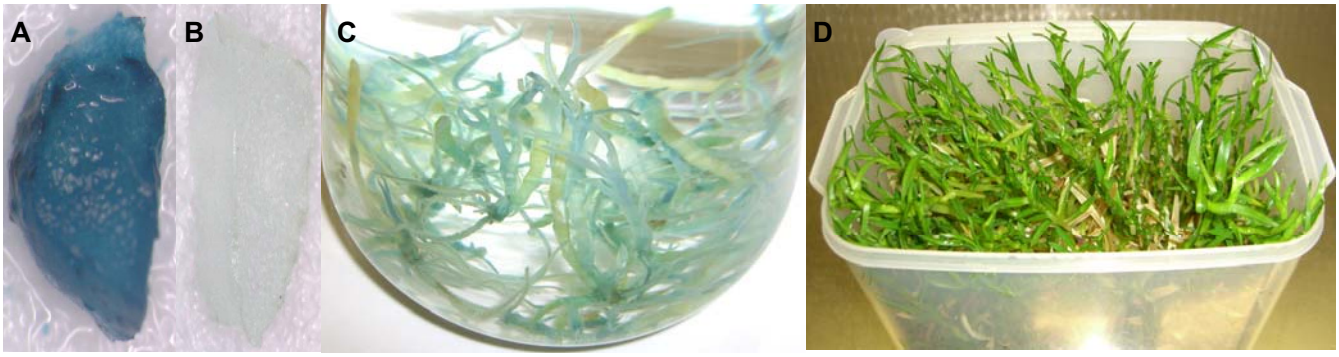

Fig. 1 Stable GUS expression in *Cymbidium* Twilight Moon 'Day Light'. T_3 pWI-GUS half-PLB (**A**) and transformed plants (**C**) 24 months following particle bombardment (**D**) and growing on selective VW_{PLB} medium containing 100 mg L^{-1} kanamycin. (**B**) A control, untransformed PLB sliced in half.

No. 1 filter paper in a circular area of 3 cm in diameter in Petri dishes overlaying VW_{PLB} or VW_{CALLUS} medium). Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's new multiple range test (DNMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Hybrid *Cymbidium* expressing transient and stable transgenic GUS PLBs or plants (**Fig. 1**) could be obtained when either PLBs or callus were used as the initial starting material. The bombardment conditions were optimized according to Teixeira da Silva and Tanaka (2009c) for *Cymbidium* since the level of transient transgene expression will impact stable transgene expression, as observed with ornamental chrysanthemums (Teixeira da Silva and Fukai 2002).

The ideal conditions established for callus and/or PLB transformation in this study were: delayed selection (1 week after bombardment), osmoticum pre-treatment of plant material (0.3 M raffinose), choice of plasmid (pWI-GUS), and age and developmental stage of plant material (1-week-old PLBs) (**Table 1**). Osmotic pre-treatment with a high concentration of an osmoticum to the medium used in particle bombardment can increase transformation efficiency (TE) (Sanford *et al*. 1993). The addition of mannitol or sorbitol has shown to increase the TE of bombarded tobacco (Russel *et al*. 1992) and maize (Vain *et al*. 1993; Kemper *et al*. 1996) and an estimated 30 other crops. A mixture of mannitol and sorbitol also increased the TE of *Hypericum perforatum* cell supension cultures bombarded with pCAMBIA 1301 (Franklin *et al*. 2007) and was also used to maximize creeing bentgrass TE of chitinase, glucanase, and bar genes (Wang *et al*. 2003). High concentrations (30 or 60 mM) of ammonium nitrate did not increase TE in hybrid *Cymbidium*, unlike what was reported for *Triticum aestivum* (Greer *et al*. 2009) (data not shown). In orchid tissue culture, osmotic agents are commonly employed to increase somatic embryogenesis e.g., *Oncidium* (Jheng *et al*. 2006).

Orchid transformation was first achieved in the genera *Vanda* (Chia *et al*. 1990, 1994) and *Dendrobium* (Kuehnle and Sugii 1992) using particle bombardment. The first example of *Agrobacterium*-mediated transformation in orchids was only a few years later for *Dendrobium* (Nan *et al.* 1998).

In particle bombardment, varying gas pressure had no substantial effect on TE in *Dendrobium* (Nan and Kuehnle 1995), but did affect TE significantly in other genotypes (Tee and Maziah 2005). The size of gold particles used generally affected the TE in orchid transformation studies. Au particles of 0.6 µm were most efficient for gene delivery into *Cymbidium* (Yang *et al*. 1999), while those of 1.0 µm resulted in increased transient expression of genes inserted in *Dendrobium* compared to particles 1.6 µm in size (Tee and Maziah 2005). In this study TE was measured either as the level of GUS expression in PLBs derived from particle bombardment with pBI121 and pWI-GUS and selection on

Table 1 Effect of different particle bombardment conditions on *Cymbidium* Twilight Moon 'Day Light' PLB and/or callus transient transformation efficiency using previous optimized parameters as basal conditions (Teixeira da Silva and Tanaka 2006, 2009c).

Trial No.	Conditions	Explant survival (%)	No. GUS spots/PLB explant	Other notes
1: Delayed selection (time after particle bombardment)				
	Immediately after	42 b	1.46 ± 0.04 b	
	1 week after	88 a	6.13 ± 0.62 a	
	2 weeks after	96 a	4.34 ± 0.48 ab	High level of escapes (non-transgenic)
2: Osmoticum pre-treatment of plant material				
	No osmoticum*	100 a	3.16 ± 0.46 b	
	Mannitol 0.4 M + Sorbitol 0.4 M	96 a	10.01 ± 1.46 a	Some deformed PLBs (< 1%)
	Raffinose 0.3 M	98 a	12.61 ± 1.20 a	
3: Choice of plasmid				
	No plasmid*	88 a	0 c	
	pBI121	96 a	1.78 ± 0.86 b	Kanamycin resistance
	pWI-GUS	98 a	10.66 ± 2.01 a	Deformed shoots; kanamycin resistance
	pMSP38	100 a	NA	Bialaphos resistance
4: Age and developmental stage of plant material				
	1 day-old callus**	NQ	2.31 ± 1.68 c	Callus very dispersed by bombardment
	1 week-old callus**	96 a	14.21 ± 2.48 a	
	3 weeks-old PLBs	100 a	8.61 ± 2.04 b	
	2.5 months-old PLBs	100 a	0.62 ± 0.18 d	

* = control; ** = callus is considered to be undifferentiated or immature PLB clusters according to Teixeira da Silva and Tanaka (2006); NA = not applicable; NQ = not quantifiable.

Except for Trial No. 1, data scored 72 h after particle bombardment and represent the mean ± SD (standard deviation) of at three replicates of $n = 20$ each. In each column and for any ONE trial, the values with different letters are significantly different ($P \leq 0.05$) according to DNMRT (Duncan's new multiple range test) or according to the χ^2 test ($P \leq 0.05$) for percentage values.

All other particle bombardment conditions were optimized elsewhere (Teixeira da Silva and Tanaka 2009c).

Table 2 Percentage of explants (PLBs) showing a change in transgene activity over three generations based on GUS detection and survival on kanamycin-supplemented selection medium (pBI121 and pWI-GUS) or selection only on selective medium containing bialaphos or kanamycin (pMSP38). Each generation consisted of a 90-day sub-culture period, i.e. the time it takes for a *Cymbidium* PLB to mature and initiate root and shoot development.

Plasmid	T_1	T_2	T_3	Transformation efficiency (mean of $T_1 + T_2 + T_3$)
	GUS activity (any level)			
pBI121	3.3%	1.7%	1.7%	2.2%
pWI-GUS	20.0%	13.3%	23.3%	18.7%
	Kanamycin resistance			
pBI121	73.3%	33.3%	13.3%	39.9%
pWI-GUS	83.3%	68.3%	65.0%	72.2%
	Bialaphos resistance			
pMSP38	6.8%	11.6%	10.0%	9.4%
	Kanamycin resistance			
pMSP38	40.0%	26.6%	20.0%	28.8%

Data based on three replicates of $n = 20$ each. All values truncated, not rounded up.

kanamycin-containing medium (at 50 mg L^{-1} for T_1 and T_2 and then increased to 100 mg L^{-1} for T_3) with or based on selection on bialaphos-containing medium (at 5 mg L^{-1}) following bombardment with pMSP38. In all cases the basal medium was VW_{PLB} or VW_{CALLUS}, optimized for the growth of *Cymbidium* PLBs and callus, respectively (Teixeira da Silva and Tanaka 2006). As the transgene generation increased, there was in general a decrease in the level of TE (**Table 2**). Wherever an increase was observed, for example in GUS activity for PLBs bombarded with pWI-GUS or bialaphos resitance in PLBs bombarded with pMSP38, one possible explanation may be that in most cases, the entire PLB is not made up of transformed cells but rather is made up of a collection on transformed and non-transformed cell clusters or tissues, in essence chimeras. Depending on how sub-culture is performed, non-transformed tissue would be selected against and transformed tissue would survive in T_x generations as X tends to ∞. It is thus possible to islate PLBs in which all tissues are fully transgenic (i.e. **Fig. 1A**), and 24-month-old *Cymbidium* plantlets whose roots and shoots are also fully transgenic (**Fig. 1C**; in this case transgenesis measured by GUS activity).

Like gas pressure, the distance between the plant tissue and the stopping screen (in helium-driven biolistic bombardment devices) has not been conclusively shown to affect orchid TE, though some studies have reported higher TE with certain distances used. For example, Tee and Maziah (2005) found that greatest transient expression of inserted genes was obtained with the distances of 6 and 9 cm for two different types of calli. However, there was no statistically significant difference between the two distances used, which is different from another observation in which a distance of 9 cm for *Dendrobium* calli resulted in highest TE (Chai *et al*. 2007).

Several biological factors also contribute to TE. Amongst these are orchid genotype, type of plant tissue used for bombardment, and selection conditions. The particle bombardment protocols optimized by researchers for different orchid genera are significantly different, clearly demonstrating that orchid genotype has a significant impact on TE. Transformation protocols for various orchid species and hybrids can differ even within a specific genus. For example, the highest GUS transient expression for six *Dendrobium* hybrids was achieved with a bombardment helium pressure of 900 psi (Nan and Kuehnle 1995), while 1100 psi with a target tissue distance of 6 cm was found to be optimal for the transformation of *D. nobile* and *D. phalaenopsis* (Men *et al*. 2003a), and 1350 psi with a target tissue distance of 9 cm for hybrids *D*. Madame Thong-In and *D*. Chao Praya Smile (Chai *et al*. 2007). It is thus recommended that the physical parameters involved in particle bombardment should be optimized for each orchid species and hybrid used (Nan and Kuehnle 1995), though identical parameters can probably be used for hybrids with very similar genetic backgrounds, such as *D*. 'Madame Thong-In' and *D*. 'Chao Praya Smile'.

In previous particle bombardment optimization studies for *Cymbidium* (Teixeira da Silva and Tanaka 2009c), the following observations were made: i) Two shots resulted in significantly higher GUS spots or GFPs per explant (both PLBs and callus) than one shot without impacting explant mortality. ii) Gold was superior to tungsten as the microcarrier. iii) A 1100 p.s.i. rupture pressure gave significantly higher GFP values than either 900 or 1350 p.s.i. for both PLBs and callus but for callus explant mortality was higher at 1110 than at 900 p.s.i. iv) A 6-cm target distance was ideal for both explant types. v) Pre-culture period did not affect GFP formation in either explant type. vi) The trend was the same for both plasmid types (pBI121 and pWI-GUS) although the latter plasmid resulted in significant higher GFP values than the former, but with no difference in explant survival.

The orchid tissue type used in the bombardment process can also have a significant effect on TE. Nan and Kuehnle (1995) found that the highest transient GUS activity after transformation was observed for PLBs, followed by etiolated shoots and protocorms. However, as chimerism could occur if orchid embryos and protocorms are used as target tissue (Kuehnle and Sugii 1992), most studies utilize PLBs or calli for bombardment. One study identified three distinct types of calli, and reported that type B callus (light yellow, nodular, and structurally compact) had significantly greater transient GFP expression after bombardment than type A (white or transparent, slightly friable) or type C (yellow and hollow-centered) calli (Tee *et al*. 2003). Choice of tissue type used is therefore important in optimization of a particle bombardment protocol for orchid transformation.

The selection process is an integral part of any transformation protocol, and selection conditions can, to a large extent, determine the successful isolation of real transformants. In particular, selection stringency and the number of recovery days after transformation have a profound effect on TE. For example, if selection is performed using very high amounts of the selection agent such as bialaphos, putative transformants may die together with non-transformants before being selected for. On the other hand, too-low amounts of selection agent will result in numerous false positive results from 'escapes'. Timing of selection is also crucial, as plant tissues require a healing period after bombardment on medium with no selection agent to recover from the damage. No transformants are obtained when selection is performed immediately after bombardment (Chai *et al*. 2007). Delayed selection has been shown to adversely affect TE, e.g. when transformation frequency was reduced to 0% when selection was initiated 30 days after bombardment for *D. phalaenopsis*, but was as high as 14% when selection was performed 2 days after bombardment (Men *et al*. 2003).

Both *uid*A and *bar* transgenes could be successfully promulgated into at least the T_3 generation without a loss in transgene (GUS) activity or resistance to bialaphos, respectively (**Table 2**). Since survival of sub-cultured PLBs was similar over the three transgene generations (i.e., T_1 to T_3), data is not presented in **Table 2**.

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